

Remarks

Claims 1-17 were pending in the subject application. By this Amendment, Applicants have amended claims 1-4 and 15 and added claims 18-36. Claims 5-14 and 17 have been canceled. Entry and consideration of the amendments presented herein is respectfully requested. Accordingly, claims 1-4, 15, and 18-36 are currently before the Examiner for examination and support for the newly presented claims can be found, for example, at pages 58-65 of the subject application. Favorable consideration of the pending claims is respectfully requested.

In paragraph 4 of the Office Action of January 31, 2003, the Patent Office has objected to the specification and drawings as including sequences not identified by SEQ ID NO: in either the specification, figures, or description of the figures. The Office Action further requested correction of the specification to attend to this error. Applicants have amended the subject specification to include a "Brief Description of the Sequences" for the newly added sequences 7-13. The description of the Figures has also been amended. A replacement sequence listing in computer readable format and on paper is being submitted with this Amendment. I hereby certify that the paper and computer readable copies contain the same information and that no new material is added by this submission. Entry and consideration of the Sequence Listing is respectfully requested. Applicants respectfully submit that this issue has been attended to in this response and respectfully request withdrawal of the objection.

Claims 1-4 are rejected under 35 U.S.C. §101 on the grounds that the claimed invention lacks a well-established, specific, and/or substantial utility. Claims 1-4 have also been rejected under 35 U.S.C. § 112, first paragraph, on the grounds that one skilled in the art would not know how to use the claimed invention because it lacks a well-established, specific, and/or substantial utility. Specifically, the Office Action states that the claimed invention comprising SEQ ID NO:1 wherein a "T" is present at position 12347 lacks utility. The Office Action also argues that the subject invention fails to provide any specific or substantial utility for the polymorphic nucleic acids of SEQ ID NO:1 wherein a "T" is present at position 12347 in that the specification fails to provide any association of the polymorphism with any disease state and that one skilled in the art would be required to engage in additional experimentation in order to confirm a real world use for the polymorphic nucleic acid. Applicants respectfully traverse.

It is respectfully submitted that the claimed invention has a specific and well-established utility. At the outset, it is respectfully submitted that the claimed invention is drawn to a polynucleotide comprising a contiguous span of at least 150 nucleotides of SEQ ID NO:1, wherein said contiguous span comprises a T at position 12347. SEQ ID NO: 1 contains a partial genomic sequence from chromosome 11 that comprises the regulatory regions and coding regions of the polypeptide identified as AA4RP in this application. The polynucleotide and polypeptide designated as AA4RP appear to be a human analog of the previously identified murine and rat regeneration associated protein 3 (RAP3; or human apolipoprotein-V (see, for example, Van der Vliet *et al.*, *J. Biol. Chem.*, 2001, 276:44512). The claims are not drawn to methods of associating the claimed polynucleotide sequence comprising a T at position 12347 with a disease or condition and Applicants respectfully submit that the claimed invention has uses (utilities) other than that focused upon in the Office Action.

However, it is respectfully submitted that the art recognizes the usefulness of SNPs in association with various disease conditions, including hyperlipidemia and other diseases that are taught in the specification (see, for example, page 22, lines 23-33). Šeda *et al.* (*Physiol. Res.*, 2003, 52:141-146) teach that twelve (12) SNP's have been identified in the APOA-V gene region, including at least one in the promoter region of the gene. A number of these SNPs have been associated with various conditions, including hyperlipidemia (see, for example, section designated human SNPs, pages 144-145). As indicated by Šeda *et al.*, a SNP located in the promoter region of the APOA-V gene (SNP3) is associated with significantly elevated plasma TG levels in hyperlipidemic individuals. SNP3 has also been associated with higher levels of triglycerides. Thus, it is respectfully submitted that one skilled in the art would have recognized the usefulness of the SNP at position 12347 of SEQ ID NO: 1 for the assessment of such conditions in individuals, particularly in view of the teachings of the specification that associate elevated levels of AA4RP with obese individuals or individuals on a high fat diet (see specification, page 15, lines 27-30).

Applicants further submit that one skilled in the art would have recognized a specific and credible utility for the claimed invention in view of the high degree of homology of the AA4RP polypeptide with rat RAP3. As indicated in the specification, AA4RP is a homolog of rat RAP3 (see page 3, lines 16-17) and elevated serum levels of RAP3 are observed after liver damage. Thus, it is

respectfully submitted that one skilled in the relevant art would have recognized that the claimed polynucleotide sequence would have been useful in the expression of AA4RP and AA4RP would have been useful in the production of antibodies thereto. These antibodies, in turn, would be expected to be useful in the identification/diagnosis of liver related disorders or liver damage as taught in the specification, (see page 103, lines 25-33). Van der Vliet *et al.* (*J. Biol. Chem.*, 2001, 276:44512) teach that RAP3 levels elevate in response to liver damage in mice and humans (see page 44519, column 1, first full paragraph).

Additionally, it is respectfully submitted that one skilled in the art would have recognized other utilities for the claimed invention in view of the teachings of the specification. For example, it is respectfully submitted that the claimed invention would have been recognized as being useful in the generation of antisense oligonucleotides or triple helix tools that inhibit the expression of AA4RP (see specification, pages 126-128). Thus, it is respectfully submitted that one skilled in the art would have recognized a specific, credible, and well established utility for the claimed invention and that one skilled in the art would have been able to use the invention in view of such teachings. Accordingly, reconsideration and withdrawal of the rejection of claims 1-4 under 35 U.S.C. §§ 101 and 112, first paragraph, is respectfully requested.

Claims 1-4 have been rejected under 35 U.S.C. § 112, first paragraph, on the grounds that the specification fails to provide an adequate written description of the claimed invention and that it is unclear that the inventors, at the application was filed, were in possession of the claimed invention. As indicated in the Office Action, the claims are broadly drawn to any nucleic acid comprising at least 8 contiguous nucleotides of SEQ ID NO: 1 or a nucleic acid that encodes at least 6 consecutive amino acids of SEQ ID NO: 3. The Office Action asserts that the claimed invention lacks adequate written description in that specification has not provided a representative number of species that encode the recited number of contiguous nucleotides. Applicants respectfully traverse.

Applicants submit that the as-filed sequence listing provides adequate written description for polynucleotides, such as those claimed herein. For example, the sequence listing would allow one skilled in the art to identify a multitude of polynucleotides that comport with the limitations presented in the claims; such claims require a contiguous span of at least 150 nucleotides of SEQ ID NO: 1 that contain a biallelic marker as identified in the specification. Likewise, it is respectfully

submitted that the sequence listing would also allow one skilled in the art to identify a variety of polynucleotides that encode a polypeptide . Accordingly, reconsideration and withdrawal of the rejection are respectfully requested.

Claims 1-4 have been rejected under 35 U.S.C. §102(b) as anticipated by Armentano *et al.* It is respectfully submitted that the reference fails to teach an isolated, purified, or recombinant polynucleotide comprising a contiguous span of at least 150, 200, 500, 1000, 2000, 5000, 10000, or 50000 nucleotides of SEQ ID No 1 or the complements thereof, wherein said contiguous span comprises a T at position 12347 of SEQ ID No 1. Accordingly, reconsideration and withdrawal of the rejection is respectfully requested.

Claims 1-2 have been rejected under 35 U.S.C. §102(b) as anticipated by Waters *et al.* It is respectfully submitted that the reference fails to teach an isolated, purified, or recombinant polynucleotide comprising a contiguous span of at least 150, 200, 500, 1000, 2000, 5000, 10000, or 50000 nucleotides of SEQ ID No 1 or the complements thereof, wherein said contiguous span comprises a T at position 12347 of SEQ ID No 1. Accordingly, reconsideration and withdrawal of the rejection is respectfully requested.

Claims 1-2 and 15 have been rejected under 35 U.S.C. §102(a) as anticipated by Hattori *et al.* It is respectfully submitted that the reference fails to teach an isolated, purified, or recombinant polynucleotide comprising a contiguous span of at least 150, 200, 500, 1000, 2000, 5000, 10000, or 50000 nucleotides of SEQ ID No 1 or the complements thereof, wherein said contiguous span comprises a T at position 12347 of SEQ ID No 1. Hattori *et al.* also fail to teach a polynucleotide providing the limitations of claims 18-36. Accordingly, reconsideration and withdrawal of the rejection is respectfully requested.

Claim 15 has been rejected under 35 U.S.C. §102(a) as anticipated by Chamuleau *et al.* Applicants respectfully submit that the reference fails to anticipate the claimed invention. For example, the reference fails to teach the limitations of claims drawn to polynucleotides as claimed in claims 18-36.

Claim 15 has been rejected under 35 U.S.C. §102(b) as anticipated by Marra *et al.* or Shaikh *et al.* Applicants respectfully submit that the reference fails to anticipate the claimed invention. For

example, the reference fails to teach the limitations of claims drawn to polynucleotides as claimed in claims 18-36.

It should be understood that the amendments presented herein have been made solely to expedite prosecution of the subject application to completion and should not be construed as an indication of Applicants' agreement with or acquiescence in the Examiner's position. Applicants expressly reserve the right to pursue the invention(s) disclosed in the subject application, including any subject matter canceled or not pursued during prosecution of the subject application, in a related application.

In view of the foregoing remarks and amendments to the claims, Applicants believe that the currently pending claims are in condition for allowance, and such action is respectfully requested.

The Commissioner is hereby authorized to charge any fees under 37 C.F.R. §§1.16 or 1.17 as required by this paper to Deposit Account No. 19-0065.

Applicants invite the Examiner to call the undersigned if clarification is needed on any of this response, or if the Examiner believes a telephonic interview would expedite the prosecution of the subject application to completion.

Respectfully submitted,



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Attachments: New pages 1-45 (Sequence Listing) of the subject specification; Sequence Listing in computer readable format; copy of Šeda *et al.*

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MINIREVIEW

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## New Apolipoprotein A-V: Comparative Genomics Meets Metabolism

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### Summary

The availability of the human genome sequence and the recently completed draft sequences of two major mammalian model species, the mouse (*Mus musculus*) and the rat (*Rattus norvegicus*), allow researchers to apply novel approaches for gene identification and characterization, using methods of comparative and functional genomics. Recently, a new gene coding for apolipoprotein A-V was identified in the vicinity of APOA-I/C-III/A-IV cluster on human chromosome 11q23 by comparative sequencing method. In a relatively short time, compelling evidence accumulated for the substantial role of APOA-V in lipid metabolism. Studies in knock-out and transgenic mice revealed that its expression pattern correlates negatively with triglyceride levels. This observation was verified in human population studies in variety of ethnic and age groups. Several single nucleotide polymorphisms were described and particular SNP alleles and haplotypes in the APO A-V gene region were shown to be associated with dyslipidemia. The discovery and characterization of the APO A-V demonstrates current possibilities of the integrative approaches in biology, boosted by the available bioinformatic tools.

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### Key words

Apolipoprotein A-V • Comparative genomics • Triglyceride • Genetic models • SNP

### Introduction

The use of animals as models for human disease is not a recent issue, particularly mice and rats have served as physiological and pharmacological models since the 19th century (recently reviewed by Jacob and Kwitek 2002). Nowadays, the availability of the draft sequence of human, mouse and rat genomes and the genome projects of other model organisms rapidly

progressing to completion, it is possible to study the molecular aspects of pathological traits on the whole-genome level and even in comparison among multiple species. An emerging field, termed *comparative genomics*, involves by definition the analysis of two or more genomes in order to identify the extent of similarity of various features, or a large-scale screening of a genome to identify sequences present in another genome. Applications range from the identification of genes and

regulatory sequences to the study of evolutionary relatedness of species (Strachan and Read 1999).

On the other hand, *functional genomics* refers to large-scale investigation of gene function and annotation of the physiological information to the respective genome. Integration of the functional and comparative approaches *via* robust bioinformatic applications allows cross-referencing various classes of genomic information among species. The ultimate goal of this process is the identification of new genes and functions that may help in deciphering the molecular basis of disease in man. Eventually, the acquired knowledge will provide fundamental information for the production of therapeutics that would causally target the underlying pathological processes. One of the areas currently capitalizing on the recent progress of genomics and concomitant development of bioinformatic tools is the research of lipid metabolism.

Plasma lipids constitute an important factor acting as a determinant of susceptibility to various common classes of disease, cardiovascular disease and atherosclerosis being on the top of the list (reviewed by Steinberg and Gotto 1999). Dyslipidemias *per se* constitute the enhanced risk for manifestation of such conditions. Furthermore, they tend to cluster with other unfavorable metabolic states, like hypertension, obesity or insulin resistance (Reaven 1995). Although there are plenty of factors contributing to individual plasma lipid levels, the apolipoproteins (apoproteins) represent a major component of lipid metabolism dynamics. At present time, the apolipoproteins A to M have been identified and the genes coding for most of them were assigned to their genomic loci. As shown in Figure 1, there are two major clusters of apolipoprotein genes on human chromosomes 11 and 19.

## Apolipoproteins in Human Genome

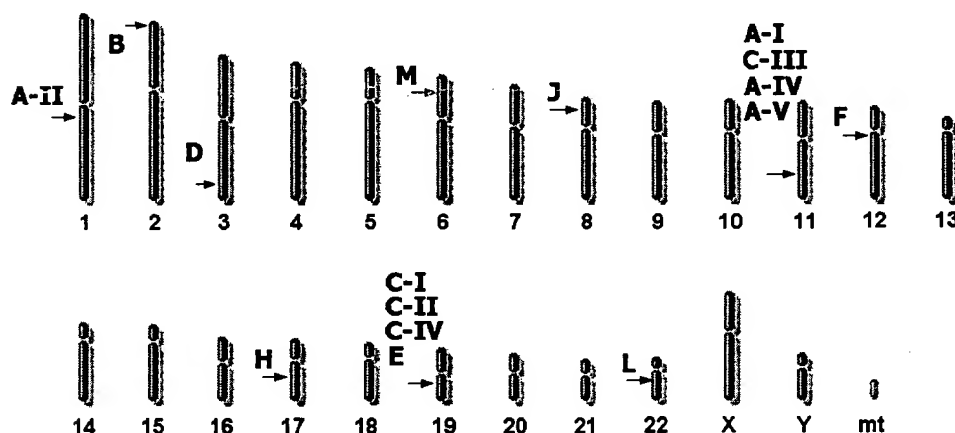


Fig. 1. Chromosomal localization of the human apolipoprotein genes.

The apolipoprotein gene cluster APOA-I/C-III/A-IV on human chromosome 11q23 has been thoroughly studied and has been linked to defects in lipid metabolism both in humans and model organisms (reviewed by Groenendijk *et al.* 2001). Recently, two independent research groups identified a novel apolipoprotein in the vicinity of this cluster, and designated it apolipoprotein A-V (MIM 606368). The stories of its discovery nicely depict recent possibilities of comparative genomics.







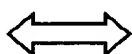
### Two routes to discovery

#### Rat – early phase of liver regeneration

Using method of cDNA subtractive hybridization, van der Vliet *et al.* (2001) identified three novel upregulated genes in regenerating rat liver after 70 % hepatectomy, designating them regeneration associated proteins (RAP) 1, 2 and 3. After obtaining and sequencing the full-length cDNA of RAP3 from rat liver cDNA library, database

search revealed its 90 % homology with murine expressed sequence tag clones (EST) clones of the mouse liver and fetus and 80 % homology with part of the human chromosome 11q23, where the APOA-I/C-III/A-IV cluster resided. Predicted protein was compared to APOA-I and A-IV (and displayed 20-28 % homology).

Using polyclonal antibody raised against recombinant RAP3, authors identified RAP3 in plasma of rats regardless of the undergone hepatectomy. Given the similarity with other apolipoproteins and its presence in the HDL lipid fraction, the novel gene was designated ApoA-V.

	<b><i>ApoC-III</i></b>		<b><i>ApoA-V</i></b>	
	<b>k.o.</b>	<b>o.e.</b>	<b>k.o.</b>	<b>o.e.</b>
<b>TG</b>				
<b>CH</b>				<b>?</b>

**Fig. 2.** Summary of the effects of knock-out (k.o.) or overexpression (o.e.) of *ApoC-III* and *ApoA-V* genes on serum levels of triglycerides (TG) and cholesterol (CH). In case of the overexpression of *ApoA-V*, there are ambiguous results so far (see text).

#### Mouse and human – comparative sequencing

Focusing on neighborhood of human APOA-I/C-III/A-IV cluster, Pennacchio *et al.* (2001) sequenced 200kb of orthologous mouse DNA and compared the mouse and human sequences. A region with substantial interspecies conservation of nucleotide sequence (CNS) was found, containing a putative apolipoprotein-like (APOA-V) gene (the visual representation of the alignment of the APOA-I/C-III/A-IV/A-V region among seven different species is available at [http://pga.lbl.gov/cgi-bin/get\\_gene?id=246](http://pga.lbl.gov/cgi-bin/get_gene?id=246)). Existence of matching ESTs from publicly available databases suggested the gene was transcribed. The predicted protein (sequence of 368 amino acids) showed strongest similarity to mouse ApoA-IV (24 % identity and 49 % similarity). Furthermore, the protein structure analysis revealed characteristic features of lipid-binding apolipoproteins. Expression profile in human and mouse tissues showed the gene was expressed predominantly in

liver. Genetically modified mice lacking ApoA-V were derived and found to have four-fold increase in plasma triglycerides (TG), contrasting with the transgenic mice overexpressing *human* APOA-V with ~66 % decrease in plasma TG, providing direct evidence for the role of APOA-V in triglyceride metabolism. Subsequently, authors identified 4 single nucleotide polymorphisms (SNPs) surrounding the human APOA-V locus. They demonstrated in two independent cohorts that minor allele of SNPs 1 a 3 were associated with high triglyceride levels (details in sections "Human SNPs" and "Haplotype studies").

#### Further studies in rodents

Since ApoA-V knockout mice were shown to be hypertriglyceridemic, van der Vliet *et al.* (2002) generated mice with adenoviral overexpression of *mouse* ApoA-V. As in mice overexpressing *human* APOA-V, elevated serum ApoA-V levels were associated with a



substantial (six-fold) decrease of serum triglycerides. Unexpectedly, all lipoprotein fractions were found to have a reduced cholesterol content, resulting in reduction of total cholesterol levels by ~40 %. The discrepancy with normal cholesterol levels of *human* APOA-V overexpressing mice was attributed to a much higher

expression of ApoA-V in the model used. Both studies with transgenic and knock-out mice showed consistently an effect of ApoA-V on lipid levels opposite to that observed in mice overexpressing or lacking ApoC-III, respectively (Fig. 2).

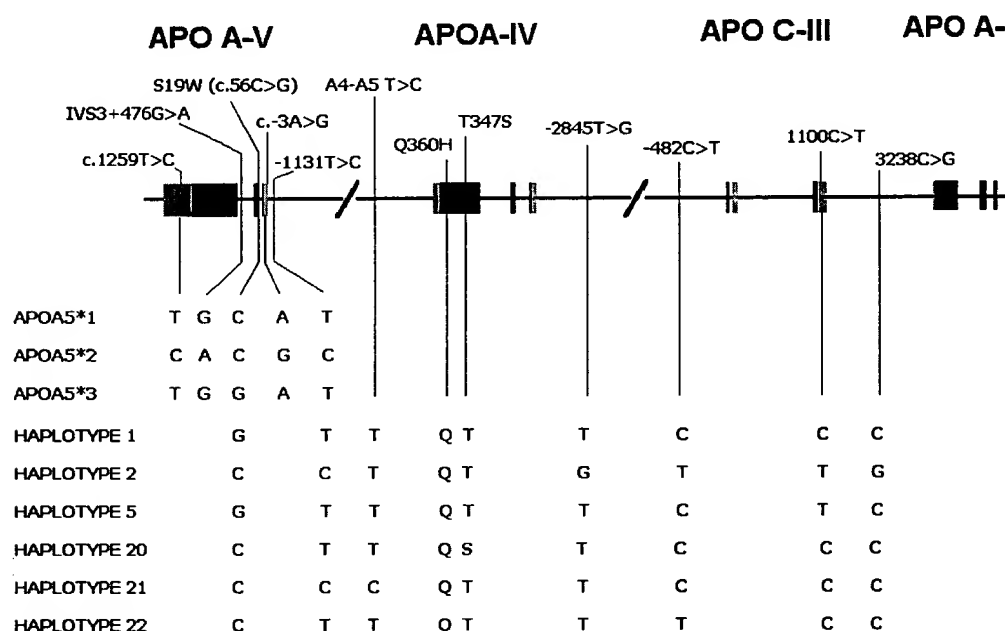


Fig. 3. Summary of haplotype studies in the apolipoprotein cluster. APOA5\*1 -3 are haplotypes designated by Pennacchio *et al.* (2002), selected haplotypes from the total of 22 are shown according to Talmud *et al.* (2002) (haplotypes 1,2 and 5 represent group with highest TG levels, conversely, the carriers of the haplotypes 20, 21 and 22 displayed the lowest TG levels). Dark boxes represent exons, light boxes represent untranslated regions of the genes.

### Human SNPs

In the original paper, Pennacchio *et al.* (2002) described four SNPs within the APOA-V gene region or its close vicinity, designating them SNP 1-4. The first three of them were shown to be in a strong linkage disequilibrium, suggesting the existence of common haplotype in APOA-V region influencing plasma TG levels. To date, 11 SNPs are annotated in publicly available databases (e.g. <http://www.ncbi.nlm.nih.gov>, NCBI). So far, the SNP3 (-1131T>C) and S19W (serine/tryptophan) polymorphisms received most attention of several research groups.

The discovery of APOA-V and the fact that familial combined hyperlipidemia (FCHL) has been

repeatedly associated with APOA-I/C-III/A-IV cluster prompted a study in which Ribalta *et al.* (2002) used SNP3 in the APOA-V promoter region as the genetic marker to search for associations between the APOA-V and TG metabolism in group of 16 FCHL families (n=103), contrasting them with normolipidemic Dutch group (n=89) and population-based Spanish control group (n=408). APOA-V seemed to be associated with TG-related variables only in FCHL group without adjustment for confounding factors (age, gender, body mass index) and the TG levels even in carriers of the minor -1131C allele were relatively low ( $1.82 \pm 1.33$  mmol/l,  $1.49 \pm 1.06$  mmol/l and  $0.90 \pm 0.42$  mmol/l in FCHL and the two control groups, respectively). However, when the association was evaluated in normolipidemic and

hyperlipidemic individuals within the 16 FCHL families separately, carriers of the rare allele C/C displayed significantly increased plasma TG concentrations. Moreover, this APOA-V variant was present more often in FCHL patients and their relatives. Authors therefore concluded that APOA-V acts as modulator of TG concentrations only when there is altered genetic or metabolic background and can be considered a predisposing factor for FCHL. Of interest is the reported significant interaction between APOA-V and APOC-III, confirming the previously shown negative linkage disequilibrium between the two markers.

Very recently, two studies performed in Japanese populations assessed the association of the SNP3 allele with triglyceridemia and other aspects of lipid metabolism. These studies have found significant association of the SNP3 minor (C/C) allele with higher levels of triglycerides in both the general population (Nabika *et al.* 2002) and the cohort of 552 school children (Endo *et al.* 2002), confirming the importance of this polymorphism in a non-Caucasian population. In another recent study of Czech cohort of 1142 men and 1181 women, the SNP3 C/C allele was also found to be associated with high TG in both sexes. Although the S19W polymorphism showed significant effect only in women, carriers of minor (W19) allele had higher triglyceridemia (Hubáček *et al.* 2002). Moreover, homozygous carriers of either allele were found to be more prone to the myocardial infarction. However, since there are no data on biological significance of SNP3 (no obvious transcription factor binding sites could be identified), the association of SNP3 minor allele with high TG levels may be just a marker of its linkage disequilibrium with another functional site (e.g. APOC-III -482C>T allele within an APOC-III insulin response element, as discussed below) within this undoubtedly important region in terms of lipid metabolism.

### Haplotype studies

Actually three haplotypes are described in the APOA-V gene region, designated APOA5\*1, 2 and 3. Pennacchio *et al.* (2001) showed in the first study that the minor haplotype APOA5\*2 defined by SNPs1, 2 and 3 was associated with plasma triglyceride levels in two independent cohorts (500 unrelated Caucasian men and another group of Caucasian men and women) with no concurrent effect of *SstI* polymorphism in APOC-III gene. In a follow-up study (Pennacchio *et al.* 2002), new haplotype APOA5\*3 (Fig. 3) was found to differ from the

common (APOA5\*1) variant in a G/C substitution, leading to non-synonymous substitution of tryptophan for serine (S19W). The frequency of this allele was not different between Caucasians (0.06) and African Americans (0.07), but was substantially higher in Hispanics (0.15). The APOA5\*3 haplotype was significantly more common among men and women with high plasma triglyceride concentrations and was shown to be systematically associated with increased triglyceridemia in men and women from three different ethnic groups (Caucasian, African-American, Hispanic), under three different dietary regimens and was independent of the effect of APOA5\*2 haplotype.

Talmud *et al.* (2002) carried out an elegant study focusing on the whole APOA-I/C-III/A-IV/A-V cluster. They assessed the strength of linkage disequilibrium (LD) across this region of human chromosome 11 using nine SNPs (Fig. 3) in a set of 2808 men (NPHSII study), confirming strong LD between APOA-V and APOC-III. The homozygous carriers of APOA-V rare alleles in SNP1 and S19W were shown, in consent with previous findings, to have significantly elevated TG levels. In order to discern whether these effects are independent or reflect the strong LD with APOC-III (or vice versa), haplotype analysis was performed. Twenty-two haplotypes out of 512 theoretical combinations were present in more than 10 individuals from the cohort. Significant differences were found among TG levels and independent effect of APOA-V W19 and APOC-III -482T rare "TG raising" alleles was ascertained (they were found separately in the groups with highest TG, i.e. haplotypes 1, 2 and 3 (Fig. 3).

The above mentioned haplotype APOA5\*3 corresponded to the haplotype 1, i.e. the one with the highest triglyceridemia. On the other hand, authors suggested that the effect of the APOA5\*2 haplotype was probably due to its strong LD between APOA-V -1131C and the APOC-III -482T "TG raising" allele and does not represent a functional change by itself.

In summary, considerably large amount of data have accumulated over a relatively short period from the APOA-V discovery, establishing this novel gene as an important player in lipid (mostly triglyceride) metabolism. The identification of the gene itself was facilitated by employing the current methods of comparative genomics, integrating and processing the genomic information from different species with the available arsenal of bioinformatic tools. It is presumable that studies dissecting the exact function of APOA-V within the metabolic networks will shortly follow as our

knowledge in this respect is largely hypothetical. The methods of functional and comparative genomics, particularly in defined genetic models, will also prove themselves to be invaluable tools.

### Acknowledgements

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### Reprint requests

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